Adenosine 3'5'-monophosphate phosphodiesterase of buffalo spermatozoa

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Summary. The cyclic AMP–phosphodiesterase (EC 3.1.4.17) of buffalo spermatozoa is distributed in the head, mid-piece and tail fractions and has multiple forms, 70% of which is in the bound form. The bound enzyme was not solubilized by Triton X-100, lubrol or hyamine 2389. Kinetic measurements of the soluble enzyme showed two apparent K_m values for low and high cAMP concentrations, i.e. 4.5 and 100 μM with V_{max} values of 0.25 and 2.0 nmol cAMP hydrolysed min^{-1} mg protein $^{-1}$. The bound enzyme had an apparent K_m of 66.6 μM with a V_{max} of 0.75 nmol cAMP hydrolysed min^{-1} mg protein $^{-1}$. The pH for optimum enzyme activity was 7.5 and Mg^{2+} was essential for the activity of the soluble and bound enzymes. Methyl-xanthines, ATP, ADP and PPi inhibited the soluble and bound enzymes, ATP being the most potent inhibitor.

Introduction

Cyclic nucleotides and certain inhibitors of cyclic nucleotide phosphodiesterase have been shown to induce and prolong motility in spermatozoa (Garbers, Lust, First & Lardy, 1971b; Garbers, First, Sullivan & Lardy, 1971a; Garbers *et al.*, 1973). We have previously described an enzymic system for the synthesis and degradation of cyclic AMP in buffalo, bull and goat spermatozoa (Chaudhry & Anand, 1975) and found that phosphodiesterase activity in all 3 species exceeded that of adenylate cyclase by several fold. Similar conclusions have also been reached for ram spermatozoa (Tash, 1976). Despite the marked disparity in the activities of adenylate cyclase and cyclic phosphodiesterase, a transient accumulation of cAMP occurs. For this to happen, phosphodiesterase may either be compartmentalized or occur in the cell largely in the inhibited state. The present studies were undertaken to investigate which of these possibilities was correct.

Materials and Methods

Preparation of extract

Semen was collected from Murrah buffalo bulls by using an artificial vagina. The spermatozoa (1×10^{10} cells) were washed free of seminal plasma with Krebs–Ringer phosphate buffer, pH 7.5, at room temperature 4 times, and then suspended in 100 mM-Tris–HCl buffer, pH 7.5, containing 1 mM-mercaptoethanol and 1 mM- MgSO_4 . The suspension was cooled in ice and sonicated at 2–4°C in a Branson Sonifier Model B-12 at 70 V for 3 min. The sperm homogenate was centrifuged at 100 000 g for 1 h and the clear supernatant was decanted and dialysed against the same buffer for 4 h in the cold. The particulate fraction was reconstituted to the original volume of sperm suspension with Tris–HCl buffer and the suspension was used for enzyme activity. To solubilize the enzyme activity, detergents were added to the sperm suspension to the desired concentration and sonication was carried out as described above.

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Intracellular distribution

Buffalo semen was washed twice at 22°C with 0.25 M-sucrose and once with buffered sucrose (0.25 M-sucrose containing 40 mM-Tris-HCl, pH 7.4). The washed sperm pellet was suspended in buffered sucrose, gradually cooled to 4°C and then subjected to ultrasonic vibrations in a Branson Sonifier at 50 V for 1 min. The heads, midpieces and tails were separated by differential centrifugation by the method described by Mohri, Mohri & Ernster (1965). The purity of the isolated fractions was determined by specific staining and microscopic observations. The head fraction was stained according to the method of Feulgen & Rossenbeck (1924), the midpiece by a mixed dye of 0.4 ml Janus green B (0.5%) with 3 ml 0.05% neutral red solution in ethyl alcohol, and tails were examined unstained under the microscope. Essentially pure uncontaminated preparations of the three organelles were used to study the intracellular distribution of cyclic nucleotide phosphodiesterase.

Cyclic AMP-phosphodiesterase assay

The assay mixture contained 62 mM-Tris-HCl buffer, pH 7.5, 2 mM-MgSO₄, 0.4 mM-[8-³H]cAMP (sp. act. 26 Ci/mmol: Radiochemical Centre, Amersham, U.K.) and the enzyme preparation in a final volume of 0.4 ml. After incubation for 15 min at 37°C, the reaction was terminated by placing the tubes in a boiling water bath for 2 min. The tubes were cooled to 30°C and 0.1 ml of the venom of *Crotalus atrox* (1.0 mg/ml: Sigma) was added as a source of 5'-nucleotidase to convert 5'-AMP to the corresponding nucleoside. After another 10 min of incubation at 37°C, the reaction was terminated by immersing the tubes in an ice-common salt mixture. The contents were applied to a column of Dowex-1-X8 (0.5 × 2 cm), 200–400 mesh (Sigma). Adenosine was eluted with water in the first 5 ml and the radioactivity of an aliquot in 10 ml Bray's solution (Bray, 1960) was counted in a Packard liquid scintillation spectrometer.

All chemicals were of reagent grade.

Results

Solubilization of cyclic phosphodiesterase

The recovery of enzyme activity in the 100 000 g supernatant and particulate fractions of the buffalo sperm sonicate was 10–20% higher than the total activity of cyclic phosphodiesterase estimated. Between 30 and 40% of phosphodiesterase activity was solubilized whereas 70–80% was associated with the particulate fraction. The specific activities of the soluble and bound enzymes (6.9 and 5.4 nmol cAMP hydrolysed min⁻¹ mg protein⁻¹) were higher than that of sperm sonicate (4.3 nmol min⁻¹ mg protein⁻¹). Sonication of the sperm suspension in the presence of Triton X-100, lubrol and hyamine were attempted to solubilize the total activity. Triton X-100 or lubrol (1–5%) solubilized 40% of the total enzyme activity. The cationic detergent, hyamine 2389, was comparatively more effective because treatment of the sperm suspension with 5% of the detergent extracted 55–60% of the enzyme activity. However, activity was inactivated within 24 h. The results of a typical experiment on the solubilization of phosphodiesterase activity with the three detergents is presented in Table 1.

Properties of soluble and bound cyclic phosphodiesterase

The hydrolysis of cAMP was linear for 20 min with less than 10% of the substrate hydrolysed by soluble and bound enzymes at pH 7.5, the optimum pH. The presence of Mg²⁺ was essential for enzyme activity. Kinetic measurements of the relation between enzyme activity and substrate concentration showed that cyclic nucleotide phosphodiesterase in buffalo

spermatozoa occurs in multiple molecular forms. The soluble enzyme had apparent K_m values of 4.5 and 100 μM with velocity maxima of 0.25 and 2.0 nmol cAMP hydrolysed min^{-1} mg protein $^{-1}$. For the bound enzyme, the apparent K_m was 66.6 μM and V_{max} was 0.75 nmol cAMP hydrolysed min^{-1} mg protein $^{-1}$ (Text-fig. 1).

Table 1. Solubilization of buffalo sperm cAMP-phosphodiesterase activity by detergent treatment

Treatment	Final conc. (w/v)	cAMP-phosphodiesterase*			% Activity of sonicate	
		Sperm sonicate	Soluble	Bound	Soluble	Bound
None	—	74.49	23.27	58.11	31.0	78.0
Triton X-100	1%	74.23	29.12	50.18	39.0	67.6
Lubrol	5%	79.43	31.72	55.51	39.9	69.8
Hyamine-2389	5%	85.80	48.36	52.78	56.0	61.5

* Total activity expressed as nmol cAMP hydrolysed per min.

Methyl xanthines are known inhibitors of cyclic nucleotide phosphodiesterases. The inhibitory effects of two methyl xanthines, caffeine and theophylline, were compared with such compounds as may act *in vivo*. ATP, ADP, pyrophosphate and inorganic phosphate are the reactants and products of adenylate cyclase and ATPase reactions, the two enzymes connected with cAMP metabolism. ATP was the most potent inhibitor of the soluble and bound buffalo sperm phosphodiesterase (Table 2) with ADP and PPi being less inhibitory.

Table 2. Effect of inhibitors on the buffalo sperm cAMP-phosphodiesterase

Inhibitor	Conc. (mM)	% inhibition	
		Soluble	Bound
Caffeine	5	26	47
Theophylline	5	36	60
ATP + Mg^{2+}	2 + 2	58	55
ATP + Mg^{2+}	2 + 4	60	52.5
ADP + Mg^{2+}	2 + 2	19	21
ADP + Mg^{2+}	2 + 4	21	22
PPi	2	15	20
Pi	5	00	7.3
Adenosine	5	00	15.3

Titration of the soluble and bound sperm phosphodiesterases with ATP and ADP (1–10 mM) gave inhibition curves which were hyperbolic (Text-fig. 2). For ATP, the saturation level was 94% at 7 mM for the soluble enzyme and 90% at 8 mM for the bound enzyme with half maximum at 1.3 mM and 1.5 mM, respectively. For ADP, the saturation level was 55% and 53% at 7 mM for the soluble and bound enzymes, with half maximum around 2.6 mM.

Intracellular distribution

The results shown in Table 3 demonstrate that cAMP-phosphodiesterase is distributed in the head, mid-piece and tail fractions of buffalo spermatozoa. The recovery of total enzyme activity assayed at 0.4 mM and 1 μM was 92 and 94% respectively and of protein 99% relative to the sonicated suspension. The head fraction, with 58 and 45% of the total activity at high and low

Text-fig. 1. Double reciprocal plots of cAMP hydrolysis by (a) soluble and (b) bound buffalo sperm phosphodiesterase. The concentration of cAMP ranged from 1 μ M to 1 μ M. The assay was performed as described in the text.

Text-fig. 2. Inhibition of (a) soluble and (b) bound buffalo sperm cyclic phosphodiesterase by ATP (●) and ADP (○). Assay conditions were as described in the text.

Table 3. Distribution of cAMP-phosphodiesterase in buffalo spermatozoa at high and low substrate concentrations

Fraction	Protein	cAMP-phosphodiesterase					
		Total activity*		Specific activity		Activity relative to sonicate	
		1 μ M	0.4 mM	1 μ M	0.4 mM	1 μ M	0.4 mM
Sonicate	103.5	2.54	105.9	0.025	1.02	1.0	1.0
Head	62.9	1.14	61.9	0.018	0.98	0.72	0.96
Mid-piece	21.1	0.50	12.6	0.024	0.59	0.96	0.59
Tail	13.3	0.65	11.7	0.049	0.87	1.96	0.85
Supernatant	5.3	0.11	10.4	0.020	1.97	0.80	1.93

* Total activity expressed as nmol cAMP hydrolysed per min.

substrate concentrations, had the highest enzyme activity. The combined phosphodiesterase activity of mid-piece + tail at 1 μ M was twice that assayed at 0.4 mM. The position was just the opposite for the enzyme activity which was solubilized and detected in the supernatant.

Discussion

The recovery of enzyme activity in separated soluble and bound fractions was consistently higher relative to the sonicated suspension. The greater recovery in enzyme activity was not due to the detergent action because total phosphodiesterase activity was nearly equal with and without detergent treatment (Table 1). The assay of enzyme activity of soluble and bound fractions after intermixing did not show any inhibition. The reason for the higher recovery of enzyme activity therefore remains unknown. However, a large proportion of the cyclic AMP phosphodiesterase

of buffalo spermatozoa does occur in the cell in bound form. In this respect, it resembles its counterpart in ram spermatozoa but differs in that the buffalo sperm enzyme could not be solubilized, and thus appears to be tightly bound, while ram sperm phosphodiesterase is nearly completely extracted by sonication in the presence of 1% Triton X-100 (Tash, 1976). Cell bound phosphodiesterases have been reported in other cells/tissues (De Robertis, Rodriguez De Lores Arnaiz & Alberici, 1967; Amer & Mayol, 1973; Russel & Pastan, 1973; Smoake, Song & Cheung, 1974; VanInwegen, Robison, Thompson, Armstrong & Atouffer, 1975) but studies on these enzymes are few because of limited information available for solubilizing them. Tash (1976) reported two phosphodiesterase activities in ram spermatozoa while Wells & Garbers (1976) separated three enzyme activities from the extracts of sea urchin spermatozoa by DEAE-cellulose chromatography. Kinetic measurements on the soluble and bound buffalo sperm enzymes indicated the existence of multiple molecular forms, but complete solubilization and purification will be necessary to establish the precise number of isoenzymes present.

The soluble and bound enzymes were inhibited by ATP, ADP and PPI. It was possible that the adenine nucleotides chelated the available Mg^{2+} in the assay mixture and the inhibition observed was because of the non-availability of Mg^{2+} as an essential co-factor, but this was ruled out because of the similar percentage inhibition by ATP and ADP of enzyme activity with increasing concentration of Mg^{2+} (Table 2). Although there are no reports on the ATP content in buffalo spermatozoa, it has been determined as 5–10 mM in bull spermatozoa (Cascieri, Amann & Hammerstedt, 1976). At this concentration of ATP, both the soluble and bound enzymes are inhibited up to 90%. Considering the contribution of ADP and PPI, it appears likely that the cAMP-phosphodiesterase is present in buffalo spermatozoa largely in the inhibited state. Further intracellular distribution has shown that of the total activity of cAMP-phosphodiesterase assayed at low and high substrate concentrations, 45% and 23% were found in the mid-pieces and tails, respectively (Table 3). The low levels of a cAMP-phosphodiesterase in the tail are significant in view of the localization in buffalo spermatozoa of adenylate cyclase mainly in the tail fraction (Chaudhry, 1977). An intracellular compartmentalization of cAMP-phosphodiesterase and its inhibition by ATP may therefore be important features for the physiological regulation of sperm motility.

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